(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 477 885 A2**

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number: **91116303.8**

(22) Date of filing: **25.09.91**

(51) Int. Cl.5: **C07K 13/00**, C07K 15/06, A61K 37/30

(30) Priority: **28.09.90 JP 257490/90**
06.09.91 JP 227232/91

(43) Date of publication of application:
01.04.92 Bulletin 92/14

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(71) Applicant: **TAKEDA CHEMICAL INDUSTRIES, LTD.**
3-6, Doshomachi 2-chome Chuo-ku
Osaka 541(JP)

(72) Inventor: **Nakagawa, Shizue**
23-709, 6 Nankhonaka 5-chome
Suminoe-ku, Osaka 559(JP)

Inventor: **Fukuda, Tsunehiko**
10-202, 9 Oharanonishisakaidani-cho 2-chome
Nishikyo-ku, Kyoto 610-11(JP)
Inventor: **Kawase, Masahiro**
28-108, Sasabe-daidononishi
Kawanishi, Hyogo 666(JP)
Inventor: **Yamazaki, Iwao**
9-21, Hibarigaokayamate 1-chome
Takarazuka, Hyogo 665(JP)

(74) Representative: **von Kreisler, Alek, Dipl.-Chem. et al**
Patentanwälte Von Kreisler-Selting-Werner, Deichmannhaus am Hauptbahnhof
W-5000 Köln 1(DE)

(54) **Parathyroid hormone derivatives.**

(57) Disclosed are peptides and salts thereof represented by general formul

R_1-Val-Ser-Glu-Ile-Gln-Leu-R_2-His-Asn-R_3-R_4-R_5-
His-Leu-Asn-Ser-R_6-R_7-Arg-R_8-Glu-R_9-Leu-R_{10}-R_{11}-R_{12}-
Leu-Gln-Asp-Val-His-Asn-R_{13}

wherein R_1 represents Ser or Aib; R_2 represents Met or a naturally occurring hydrophobic amino acid; R_3 represents Leu, Ser, Lys or an aromatic amino acid; R_4 represents Gly or a D-α-amino acid; R_5 represents Lys or Leu; R_6 represents Met or a naturally occurring hydrophobic amino acid; R_7 represents Glu or a basic amino acid; R_8 represents Val or basic amino acid; R_9 represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R_{10} represents Arg or His; R_{11} represents Lys or His; R_{12} represents Lys, Gln or Leu; and R_{13} represents Phe or Phe-NH_2; except that simultaneously R_1 consists of Ser, R_2 consists of Met, R_3 consists of Leu, R_4 consists of Gly, D-Ala or D-Pro, R_5 consists of Lys, R_6 consists of Met, R_7 consists of Glu, R_8 consists of Val, R_9 consists of Trp, R_{10} consists of Arg, R_{11} consists of Lys and R_{12} consists of Lys.

The parathyroid hormone (1-34) analogues are useful in hormone therapy.

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BACKGROUND OF THE INVENTION

The present invention relates to novel parathyroid hormone peptide derivatives useful in hormone therapy.

Parathyroid hormone (PTH) is synthesized in the parathyroid, and plays an important role in controlling blood calcium concentrations or phosphoric acid ion concentrations by acting on the bone, the kidney and the intestine which are its target organs. PTH is a peptide hormone consisting of 84 amino acids, and the biological action thereof can be reproduced by a peptide fragment of an N-terminal (1 through 34 amino acid) portion, G. W. Tregear et al., Endocrinology 93, 1349-1353 (1973).

The amino acid sequence of the peptide fragment of the N-terminal (1 through 34 amino acid) portion of this human type PTH (this peptide fragment is hereinafter abbreviated as human PTH(1-34) or hPTH(1-34)) is as follows:

```
  1   2   3   4   5   6   7   8   9   10  11  12  13
H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-

  14  15  16  17  18  19  20  21  22  23  24  25  26
His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-

  27  28  29  30  31  32  33  34
Lys-Leu-Gln-Asp-Val-His-Asn-Phe-OH (SEQ ID NO:1)
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From the biological action of PTH, it is expected that the use of PTH as a drug will provide a drug useful for various bone diseases and the like. However, the following properties of the peptide make it difficult:

(1) The peptide is easily decomposed by various enzymes within the body;

(2) The absorption efficiency of the peptide into the body through various routes is very low; and

(3) The peptide is unstable to various physicochemical conditions such as oxidation.

In order to solve such problems and to understand the relationship between structure and activity of the above hormone, various derivatives have been synthesized for the PTH(1-34) fragment. While, such syntheses have been conducted for bovine PTH(1-34), few examples are known for the human PTH(1-34). For example in one such derivative, when the C-terminus Phe of the human PTH(1-34) is converted to Phe-NH_2, an increase in activity is observed (Japanese Patent Unexamined Publication No. 58-96052). This increase in activity is believed to be due to inhibition of carboxypeptidase which decomposes the hormone. Further, human PTH(1-34) contains two Met residues. A molecule in which these Met residues are substituted with Nle residues prevents the hormone from losing its activity due to oxidation (Japanese Patent Unexamined Publication No. 61-24598).

SUMMARY OF THE INVENTION

In order to solve the above described problems, the inventors substituted one or more amino acid residue of human PTH(1-34) by chemical synthesis. The substitutions effect the resulting molecule's resistance to various proteases, its two-dimensional structure as well as its reaction in hydrophilic/hydrophobic or ionic media. By substituting the amino acid unstable to acidic, basic or oxidation conditions with an amino acid stable to these conditions, a molecule which is an object of the present invention is synthesized. In addition, clinically effective PTH analogues have been synthesized in accordance with the present invention.

Namely, the present invention provides:

(1) a peptide represented by general formula [I] or a salt thereof:

$$R_1\text{-Val-Ser-Glu-Ile-Gln-Leu-}R_2\text{-His-Asn-}R_3\text{-}R_4\text{-}R_5\text{-}$$
$$\text{His-Leu-Asn-Ser-}R_6\text{-}R_7\text{-Arg-}R_8\text{-Glu-}R_9\text{-Leu-}R_{10}\text{-}R_{11}\text{-}R_{12}\text{-}$$
$$\text{Leu-Gln-Asp-Val-His-Asn-}R_{13} \qquad [I]$$

wherein R_1 represents Ser or Aib; R_2 represents Met or naturally occuring hydrophobic amino acid; R_3 represents Leu, Ser, Lys or an aromatic amino acid; R_4 represents Gly or a D-α-amino acid; R_5 represents Lys or Leu; R_6 represents Met or naturally occurring hydrophobic amino acid; R_7 represents Glu or a basic amino acid; R_8 represents Val or a basic amino acid; R_9 represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R_{10} represents Arg or His; R_{11} represents Lys or His; R_{12} represents Lys, Gln or Leu; and R_{13} represents Phe or Phe-NH_2. However, except that simultaniously R_1 consists of Ser, R_2 consists of Met, R_3 consists of Leu, R_4 consists of Gly, D-Ala or D-Pro, R_5 consists of Lys, R_6 consists of Met, R_7 consists of Glu, R_8 consists of Val, R_9 consists of Trp, R_{10} consists of Arg, R_{11} consists of Lys and R_{12} consists of Lys (SEQ ID NO:2).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Naturally occurring hydrophobic amino acids of R_2 and R_6 mean hydrophobic ones among amino acids which consist of natural proteins originating from animal, plant or microorganisms, and including Leu, Ile, Val, Phe and Trp. Aromatic amino acids of R_3 include Phe, β-naphthyl Ala, Trp and Tyr. D-α-amino acids of R_4 may be any D-α-amino acid, and includes D-Leu, D-Ile, D-Nle, D-Val, D-Ser, D-Ser(But), D-Abu, D-Thr, D-Nva, D-Met, D-B-naphthyl-Ala, D-Trp, D-Tyr, D-Lys, D-Lys(Fmoc), D-Phe and D-Asn. Generally, neutral amino acids are preferable including D-Ser, D-Leu, D-naphthyl Ala, D-Trp, D-Asn and D-Tyr. Basic amino acids of R_7 and R_8 include Arg, Lys, Asn and His. The substitution of the above-described groups may be at one or more positions and the substitution combination up to three positions is preferable. Accordingly, a peptide or a salt thereof of the formula [1] may simultaneously have 10 to 12 amino acids optionally selected from a group consisting of Ser for R_1, Met for R_2, Leu for R_3, Gly for R_4, Lys for R_5, Met for R_6, Glu for R_7, Val for R_8, Trp for R_9, Arg for R_{10}, Lys for R_{11}, Lys for R_{12} and Phe for R_{13}.

Peptide synthesis in the present invention can be carried out by the use of an automatic peptide synthesizer. The method of R. B. Merrifield Advances in Enzymology 32, 221-296 (1969) applies correspondingly to a basic synthesis course. In this method, the amino acid of the carboxyl terminus is covalently bound to a resin carrier, and elimination of a protective group of an α-amino group and condensation of a protected amino acid are repeated in turn to extend a peptide chain to the amino terminus, thereby obtaining a protected peptide resin having a desired amino acid sequence. This method is based on the above-described principle. The condensation of each amino acid and the elimination of the protective groups of the α-amino groups are performed under approximately similar conditions, and purification of intermediates is not conducted. In synthesizing peptides, therefore, skill of a high order generally is not required. Moreover, the peptides are rapidly synthesized by this method, so that this method is very convenient to synthesize various peptides. The protected peptide resin thus obtained is reacted with, for example, anhydrous hydrogen fluoride, trifluoromethanesulfonic acid or trifluoroacetic acid in the coexistence of various additives, whereby elimination of the peptide from the resin and removal of all protective groups can be achieved in one step.

The resulting crude peptide can be purified by known means for purifying peptides or proteins. Examples of such means include column chromatography under various principles such as gel filtration, ion exchange chromatography using a cation exchange resin or an anion exchange resin, hydrophobic chromatography and partition adsorption chromatography, and high performance liquid chromatography.

The peptides of the present invention can be obtained in various salt forms. Examples of the salts include salts of inorganic acids, salts of organic acids such as formic acid, acetic acid, tartaric acid and citric acid, salts of inorganic bases such as sodium and ammonium, and salts of organic bases such as triethylamine, ethylamine and methylamine.

The human PTH(1-34) derivative peptides represented by general formula [I] of the present invention can be used as therapeutic agents for osteoporosis, hypoparathyroidism and hypertension. The forms thereof include injections, nasotracheal absorption agents, perrectum absorption agents, transvaginal absorption agents, percutaneous absorption agents and eye drops. In some cases, they are orally administered.

When the peptides are used as such therapeutic agents, effective amounts thereof are used to treat mammals, especially humans. Although they are generally used within the range of 1 ng to 100 μg/kg of weight, precise amounts thereof may be determined by those skilled in the art.

When the peptides are used as the therapeutic agents, they must be carefully purified so as to contain no bacteria and no pyrogens.

The peptides, when used as the therapeutic agents for osteoporosis and the like, can be administered parenterally in the form of the above-described injections, nasotracheal absorption agents, perrectum absorption agents, transvaginal absorption agents, percutaneous absorption agents or eye drops, solely or

in combination with pharmaceutically acceptable carriers, excipients or diluents. In the case of the injections, it is appropriate that the peptides are given to adults in a dose of 50 ng/kg to 5 mg/kg for 1 to 3 days, and preferably in a dose of 1 to 500 μg/kg for 1 to 3 days. For the injections, it is appropriate that the concentration of the therapeutic agent is 10 to 100 μg/ml.

When nucleotides, amino acids and the like are indicated by abbreviations in this specification, the abbreviations adopted by the IUPAC-IUB Commission on Biochemical Nomenclature or those commonly used in the art are employed. For example, the following abbreviations are used. When the amino acids are capable of existing as optical isomers, it is understood that the L-forms are represented unless otherwise specified.

Gly or G :	Glycine
Ala or A :	Alanine
Val or V :	Valine
Leu or L :	Leucine
Ile or I :	Isoleucine
Ser or S :	Serine
Thr or T :	Threonine
Cys or C :	Cysteine
Met or M :	Methionine
Glu or E :	Glutamic acid
Asp or D :	Aspartic acid
Lys or K :	Lysine
Arg or R :	Arginine
His or H :	Histidine
Phe or F :	Phenylalanine
Tyr or Y :	Tyrosine
Trp or W :	Tryptophan
Pro or P :	Proline
Asn or N :	Asparagine
Gln or Q :	Glutamine
Aib :	Aminoisobutyric acid
Nle :	Norleucine
β-Ala :	β-Alanine
hPTH :	Human PTH
Fmoc :	9-Fluorenylmethoxycarbonyl
Nva :	Norvaline
Abu :	α-Aminobutyril acid

By the amino acid substitution in the PTH(1-34) as described above, the resistance to various proteases is increased and the persistence of the activity in blood is obtained. This is achieved by, for example, substituting Aib for the 1st-position of PTH(1-34), and D-α-amino acid for the 12th-position of PTH(1-34). The position around the 12-position Gly is considered to have the β-turn structure. The substitution of a D-α-amino acid for the Gly, particularly of a bulky D-α-amino acid such as D-Leu, D-Trp or D-Val, contributes to the stabilization of this structure, and the peptide chain is prevented from being digested by a protease at this position. Substitution of naturally occurring hydrophobic amino acid for Met at 8th or 18th position of PTH(1-34) increases resistance to oxidation, and is useful in prevention of reduction or elimination of activity of the peptide. Further, the affinity of the PTH derivatives for receptors is increased and high PTH activity is expressed by the substitution of amino acid residues at other positions. For example, the 11th position of PTH(1-34) is originally Leu. However, it is more preferrable that the amino acid having an aromatic chain such as Phe, is substituted for Leu. Substitution for the 25th, 26th and 27th position basic amino acid of PTH(1-34), especially substitution of Gln or Leu for the 27th Lys; and substitution of 2-(1,3-dithiolane-2-yl)-Trp for the 23rd Trp bring about high PTH activity expression. It is understood, that the typical examples of amino acid substitution are not intended to limit the scope of the invention.

Example 1 Synthesis and Purification of PTH (1-34) Active Fragment Analogues

The peptides were synthesized in accordance with a modified method of the solid phase peptide synthesis developed by R. B. Merrifield, R. B. Merrifield, Adv. Enzymol. 32, 221-296 (1969), and an automatic peptide synthesizer 430A (Applied Biosystems) was used. Protected peptide-resins were synthesized using protocols specified by Applied Biosystems. Protected amino acid-p-oxymethyl-

phenylacetoamidomethyl resins (polystyrene-1% divinylbenzene) are used as starting materials when analogues having free carboxylic acids as carboxyl termini are desired, and 4-methylbenzhydryl resins are used as starting materials when analogues of carboxylamides are desired, and protected amino acids were condensed thereto successively. In order to protect an α-amino group of each amino acid on condensation, a tertiary-butyloxycarbonyl (BOC) group was used. Side functional groups were protected in the following manner. Hydroxyl groups of serine and threonine were protected as O-benzyl ethers, a hydroxyl group of tyrosine as a p-bromobenzyloxycarbonyl ester, carboxyl groups of glutamic acid and aspartic acid as benzyl esters, imidazole nitrogen of histidine with benzyloxymethyl, a side chain amino group of lysine with 2-chlorobenzyloxycarbonyl,a guanidine functional group of arginine with a p-toluenesulfonyl group, and indoleimine of tryptophan with a formyl group. All amino acids were obtained from Applied Biosystems Japan and Bachem Chemicals.

After all of the amino acids were condensed on the resin, the protected peptide resin was taken out of the synthesizer and dried. The peptide resin (1 g) was allowed to react with anhydrous hydrogen fluoride (8 ml) containing p-cresol (1 ml), 1,2-ethanedithiol (1 ml) and 2-mercaptopyridine (100 mg) at 0 °C for 2 hours. After completion of reaction, hydrogen fluoride was removed by distillation and the residue was washed with diethyl ether to remove most of additives. The peptide was extracted with 3% acetic acid (10 ml), and the resin was removed by filtration. The filtrate was purified by gel filtration using a Sephadex G-25 column. The conditions of gel filtration were as follows: column size: 2.8X60 cm; detecting wavelength: 230 or 280 nm; solvent: 3% acetic acid; flow rate: 40 ml/hour. Fractions containing the peptide were collected and then lyophilized. The resulting powder sample was further purified by reversed phase high performance liquid chromatography [column: YMC-pack, A-324 ODS (10X250 mm); eluting solvent A: 0.1% trifluoroacetic acid-99.9% water; eluting solvent B: 0.1% trifluoroacetic acid-99.9% acetonitrile; linear gradient elution program: 0 minute (90% A + 10% B), 30 minutes (60% A + 40% B) (if necessary another elution program may be used); elution rate: 1.6 ml/minute; detecting wavelength: 230 or 280 nm]. Peak fractions containing the desired pure product were collected, and passed through a Bio RAD AGIX8 column (acetate form, 1.8 x 5 cm). The eluate was combined with the washings, and acetonitrile was removed therefrom by distillation, followed by lyophilization. The peptides thus obtained, the result of amino acids analysis and the retention times on HPLC are shown in Table 1.

In Table 1, a and b are as follows:

a: The peptides were hydrolyzed in a tube sealed with 6 N hydrochloric acid under reduced pressure, in the presence of 4% thioglycolic acid at 110 °C for 24 hours, and then subjected to amino acid analysis. Theoretical values are designated in parentheses.

b: Names of compounds(no NH_2 at the terminus means COOH):

(1) (Leu^{18})hPTH(1-34)
(2) (Aib^{1})hPTH(1-34)
(3) (Phe^{11})hPTH(1-34)
(4) ($D\text{-}Trp^{12}$)hPTH(1-34)
(5) (Leu^{8})hPTH(1-34)NH_2
(6) ($D\text{-}Tyr^{12}$)hPTH(1-34)NH_2
(7) ($D\text{-}Ser^{12}$)hPTH(1-34)NH_2
(8) ($D\text{-}Leu^{12}$)hPTH(1-34)NH_2
(9) (3-(2-naphthyl)-$D\text{-}Ala^{12}$)hPTH(1-34)NH_2
(10) (Ser^{11})hPTH(1-34)NH_2
(11) (Phe^{11},Leu^{18})hPTH(1-34)NH_2
(12) (Leu^{8},Phe^{11},Leu^{18})hPTH(1-34)NH_2
(13) (Lys^{11})hPTH(1-34)NH_2
(14) (Phe^{11})hPTH(1-34)NH_2
(15) ($Arg^{19,21}$)hPTH(1-34)NH_2
(16) (3-(2-naphthyl)-Ala^{11})hPTH(1-34)NH_2
(17) (His^{25})hPTH(1-34)NH_2
(18) (His^{25})hPTH(1-34)
(19) (Gln^{27})hPTH(1-34)
(20) ($Arg^{19,21}$,2-(1,3-dithiolan-2-yl)-Trp^{23})hPTH(1-34)NH_2
(21) (Leu^{27})hPTH(1-34)
(22) (Lys^{11})hPTH(1-34)

c: Retention time of the peptides by high performance liquid chromatography. Analysis conditions: VISTA 5000 high performance liquid chromatography (Varian) linked to 712W autosampler (Waters) was used. Column: YMC-303 ODS (4.6x250mm); Eluent: A, 0.1% trifluoroacetic acid-99.9% water; B, 0.1%

trifluoroacetic acid-99.9% acetonitrile; Eluent concentration gradient program: 0 minute(80% A + 20% B), 30 minutes(50% A + 50% B); Flow rate 0.7 ml/minute; detective wave length 280 nm.

Table 1-1 Amino acid composition of PTH(1-34)analogues(a)

amino acid	peptide (b)					
	(1)	(2)	(3)	(4)	(5)	(6)
Asx	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
Ser	2.44(3)	1.57(2)	2.23(3)	2.45(3)	2.36(3)	2.48(3)
Glx	5.28(5)	5.30(5)	4.99(5)	5.22(5)	5.24(5)	5.30(5)
Gly	1.03(1)	1.02(1)	1.04(1)		1.02(1)	
Val	2.37(3)	2.77(3)	2.75(3)	2.87(3)	2.61(3)	2.79(3)
Met	0.98(1)	1.91(2)	1.91(2)	1.91(2)	0.93(1)	1.83(2)
Ile	0.92(1)	0.92(1)	0.89(1)	1.00(1)	0.88(1)	0.95(1)
Leu	6.53(6)	5.03(5)	4.07(4)	5.07(5)	6.19(6)	5.10(5)
Phe	1.01(1)	1.01(1)	2.02(2)	1.05(1)	1.03(1)	1.02(1)
Lys	3.09(3)	3.04(3)	3.03(3)	2.94(3)	3.07(3)	3.05(3)
His	2.80(3)	2.88(3)	2.86(3)	2.80(3)	2.80(3)	2.81(3)
Trp	0.90(1)	1.09(1)	1.06(1)	1.90(2)	0.96(1)	0.92(1)
Arg	2.00(2)	1.97(2)	1.98(2)	1.99(2)	2.02(2)	1.96(2)
Aib		1.04(1)				
Tyr						1.02(1)
HPLC retention time (minutes) c	24.2	—	—	—	24.6	24.0

Table 1-2 Amino acid composition of PTH(1-34)analogues(a)

amino acid	peptide (b)				
	(7)	(8)	(9)	(10)	(11)
Asx	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
Ser	3.39(4)	2.35(2)	2.45(3)	3.29(4)	2.07(3)
Glx	5.17(5)	5.08(5)	5.31(5)	5.14(5)	4.80(5)
Gly				1.03(1)	0.83(1)
Val	2.83(3)	2.73(3)	2.58(3)	2.55(3)	2.43(3)
Met	1.90(2)	1.90(2)	2.11(2)	2.10(2)	1.03(1)
Ile	0.94(1)	0.85(1)	0.90(1)	0.91(1)	0.92(1)
Leu	5.04(5)	5.97(6)	4.98(5)	3.92(4)	4.69(5)
Phe	1.05(1)	1.00(1)	1.07(1)	1.06(1)	1.70(2)
Lys	2.98(3)	2.93(3)	2.81(3)	2.81(3)	2.57(3)
His	2.78(3)	2.81(3)	2.67(3)	2.66(3)	2.30(3)
Trp	1.06(1)	0.86(1)	0.89(1)	0.70(1)	0.90(1)
Arg	2.01(2)	1.96(2)	1.88(2)	1.79(2)	1.61(2)
Aib					
Tyr					
HPLC retention time (minutes) c	21.9	26.4	28.1	20.8	27.1

Table 1-3 Amino acid composition of PTH(1-34)analogues(a)

amino acid	peptide (b)					
	(12)	(13)	(14)	(15)	(16)	(17)
Asx	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
Ser	2.07(3)	1.99(2)	2.51(3)	2.55(3)	2.55(3)	2.58(3)
Glx	4.83(5)	4.71(5)	4.94(5)	3.95(4)	4.98(5)	5.05(5)
Gly	1.01(1)	0.96(1)	1.01(1)	1.02(1)	1.03(1)	1.04(1)
Val	2.65(3)	2.63(3)	2.73(3)	1.80(2)	2.73(3)	2.75(3)
Met		1.66(2)	2.12(2)	2.12(2)	1.90(2)	1.91(2)
Ile	0.81(1)	0.67(1)	0.84(1)	0.86(1)	0.86(1)	0.91(1)
Leu	5.97(6)	3.92(4)	4.08(4)	5.08(5)	4.07(4)	5.08(5)
Phe	1.99(2)	1.06(1)	2.04(2)	1.04(1)	1.03(1)	1.00(1)
Lys	2.92(3)	3.76(4)	2.96(3)	2.88(3)	3.03(3)	2.00(2)
His	2.53(3)	2.45(3)	2.69(3)	2.68(3)	3.09(3)	4.07(4)
Trp	0.65(1)	0.79(1)	0.87(1)	0.86(1)	0.92(1)	0.91(1)
Arg	1.93(2)	2.21(2)	1.94(2)	3.84(4)	1.96(2)	1.91(2)
Aib						
Tyr						
HPLC retention time						
(minutes) (c)	28.4	20.8	28.1	26.6	24.8	23.4

Table 1-4 Amino acid composition of PTH(1-34)analogues(a)

amino acid	peptide (b)				
	(18)	(19)	(20)	(21)	(22)
Asx	4.00(4)	4.00(4)	4.00(4)	4.00(4)	4.00(4)
Ser	2.58(3)	2.72(3)	2.51(3)	2.99(3)	2.49(3)
Glx	5.07(5)	6.27(6)	3.92(4)	4.90(5)	5.04(5)
Gly	1.04(1)	1.00(1)	0.99(1)	1.31(1)	1.08(1)
Val	2.76(3)	2.76(3)	1.78(2)	2.77(3)	2.78(3)
Met	1.91(2)	1.91(2)	2.03(2)	1.82(2)	2.12(2)
Ile	0.89(1)	0.90(1)	0.87(1)	0.91(1)	0.91(1)
Leu	5.11(5)	5.05(5)	5.04(5)	5.90(6)	3.96(4)
Phe	1.02(1)	0.96(1)	1.04(1)	1.00(1)	1.01(1)
Lys	3.02(3)	1.91(2)	2.79(3)	1.87(2)	3.86(3)
His	3.64(4)	2.66(3)	2.64(3)	2.79(3)	2.74(3)
Trp	0.95(1)	0.84(1)	0.84(1)	0.79(1)	0.85(1)
Arg	0.98(1)	1.86(2)	3.83(4)	1.91(2)	1.90(2)
Aib					
Tyr					
HPLC retention time					
(minutes) c	24.8	26.0	28.2	29.2	23.6

Example 2 Synthesis and purification of ($Arg^{19,21}$,2-(1,3-dithiolane-2-yl)Trp^{23})hPTH(1-34)NH_2

560 mg of peptide resin synthesizing ($Arg^{19,21}$)hPTH(1-34)NH_2 was allowed to react with anhydrous hydrogen fluoride (5 ml) containing p-cresol (620 µl) and ethanedithiol(620 µl) at 0°C for 2 hours. After hydrogen fluoride was removed by distillation, the residue was washed with diethyl ether containing 0.1% 2-mercaptoethanol. The resulting product was dried and the peptide was extracted with trifluoroacetic acid (5 ml), and the resin was removed by filtration. Ether was added to the filtrate and the resulting precipitate was separated by filtration and washed with ether. 280 mg of the crude peptide was obtained. The peptide was purified by reverse phase high performance liquid chromatography. The conditions of the chromatography were as follows: Column, YMC-pack, A-324 ODS (10x250 mm); Eluent A, 0.1% trifluoroacetic acid - 99.9% water; Eluent B, 0.1% trifluoroacetic acid - 99.9% acetonitrile; Eluent concentration gradient program, 0 minute (70% A+ 30% B), 40 minutes (55% A + 45% B); flow rate: 1.6 ml/minute. Two large peaks (retention times 17.0 minutes and 18.2 minutes) were observed in the chromatography. The former peak (retention time 17.0 minutes) was recovered and changed to acetate by an ion-exchnage resin. The acetate was then lyophilized to obtain 4.9 mg of ($Arg^{19,21}$)hPTH(1-34)NH_2. After hydrolysis, the resulting product

shows the correct amino acid composition in the amino acid analysis. The ultraviolet absorption of the product shows a specific curve characteristic of a peptide comprising tryptophan.

6.9 mg of compound was obtained from the latter peak. Amino acid analysis of the compound after acid-hydrolysis showed the correct composition, but amino acid analysis after trypsin-amino peptidase M digestion showed only 0.28 residue of tryptophan and the glutamic acid was detected 0.65 residue less than the theoretical value. Ultraviolet absorption curve for the digested compound showed a peak of 289 nm and a valley of 255 nm. As a result, tryptophan side chain of the compound is deduced to be modified. The following process showed that 1,3-dithiolan linked to the C2 carbon of the side chain indole of tryptophan.

A compound (4mg) obtained from the peak at a retention time 18.2 minutes in the above high performance liquid chromatography was dissolved into 60mM sodium hydrogen carbonate pH8.0(2.6ml). TPCK-trispin(160 μg)was added to the solution and reacted for 24 hours at 37 °C, and then was inactivated by heating for 6 minutes at 100 °C. Aminopeptidese-M (0.5mg) was added to the resulting solution adjusted to pH7 and incubated at 37 °C for 24 hours and then the enzyme(0.5 mg) was further added thereto. After an additional 48 hour period, the buffer (10 ml) and the enzyme (1 mg) were added thereto and reacted for 70 hours. The resulting product was subjected to reverse phase high performance liquid chromatography to isolate a modified triptophan. Column, YMC D-ODS-5 S5 120A (20X250m); the same eluent; Eluent program, 0 minute (80%A + 20%B), 40 minutes (65%A + 35%B); Flow rate, 5ml/minute; detected at 280 nm. The isolated compound showed a maximum ultraviolet absorption at 289nm. Amino acid analysis after hydrolysis with 6N HCl containing 4% thioglycolic acid showed triptophan. When the product was subjected to high resolution FAB-mass spectrum (Nihon Denshi, Japan; AX-505W TYPE double convergence mass spectrometer), a peak at 309.0734($M+H^+$) as observed and a molecular formula $C_{14}H_{17}N_2O_2S_2$ was deduced. Further about 30 ng of the compound was subjected to ^{1}H-MNR(Nihon Denshin, JNM-GX400).

(DMSO-d_6), α-CH δ = 4.06 (1H, dd like), β-CH_2 3.54(1H, dd), 3.30(1H, dd); 1-NH 10.88 (1H); 5-CH 7.50 (1H, d); 6-CH 7.30 (1H, t like); 7-CH 7.20 (1H, t like); 8-CH 7.68 (1H, d); dithiolan 2CH 6.14 (1H, S); dithiolan 4CH_2 and 5CH_2 3.64 (2H, m) and 3.49 (2H, m).

The above data show that the isolated hPTH(1-34)NH2 analogue has 2-(1,3-dithiolan-2-yl)-tryptophan at 23th position.

Example 3 Assay of Biological Activity of PTH (1-34) Analogues

The biological activity of the peptide analogues was evaluated by a modified version of the method reported by Shigeno et al. in The Journal of Biological Chemistry 263, 18369-18377 (1988). A culture solution (Hank's solution, containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin and 0.5 mM isobutylmethyl-xanthine) containing 0.01, 0.1, 1, 10 or 100 nM analogue was added in an amount of 100 μl to a mouse cranial bone-derived osteoblast-like cell strain, MC3T3-El cells, cultivated on a 96-well multiplate (Nunclon, Nunc), followed by reaction at room temperature for 30 minutes. After addition of 100 μl of 0.2 N hydrochloric acid, the mixture was immersed in boiling water for 2.5 minutes, and cyclic adenosine monophosphate (cAMP) produced by a PTH receptor was extracted from the cells. The total cAMP in the culture solution and the cells was assayed using a commercial radioimmunoassay kit (cyclic AMP [^{125}I] kit "Du Pont-Daiichi", Daiichi Kagaku Yakuhin). An increase in cAMP production depending on the concentration of the human PTH (1-34) added as a standard was observed in each case. The biological activity of the PTH (1-34) peptide analogines is shown in Table 2.

Table 2

Biological Activity of PTH(1-34) Partial Peptides (Represented by Relative Activity to hPTH(1-34))	
hPTH(1-34)	1.00
[Leu^{18}]hPTH(1-34)	0.4
[Aib^{1}]hPTH(1-34)	1.7
[Phe^{11}]hPTH(1-34)	1.1
[Leu^{8}]hPTH(1-34)NH_2	0.5
[D-Ser^{12}]hPTH(1-34)NH_2	0.8
[D-Leu^{12}]hPTH(1-34)NH_2	0.5
[3-(2-naphthyl)-D-Ala^{12}]hPTH(1-34)NH_2	0.9
[Ser^{11}]hPTH(1-34)NH_2	0.8
[Leu^{8}, Phe^{11}, Leu^{18}]hPTH(1-34)NH_2	0.9
[Lys^{11}]hPTH(1-34)NH_2	1.1
[Phe^{11}]hPTH(1-34)NH_2	1.3
[Arg^{19,21}]hPTH(1-34)NH_2	0.9
[3-(2-naphthyl)-Ala^{11}]hPTH(1-34)NH_2	1.7
[His^{26}]hPTH(1-34)NH_2	0.9
[His^{25}]hPTH(1-34)	1.0
[Gln^{27}]hPTH(1-34)	2.5
[Arg^{19,21},2-(1,3-ditiolan-2-yl)-Trp^{23}]hPTH(1-34)NH_2	1.7
[Leu^{27}]hPTH(1-34)	1.2

Sequence Listing

SEQ ID NO:1

SEQUENCE LENGTH: 34

SEQUENCE TYPE: amino acid

TOPOLOGY: Linear

MOLECULE TYPE: Peptide

FEATURE: Partial Peptide

SEQUENCE DESCRIPTION:

Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Asn
1 5 10 15
Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp Val His
20 25 30
Asn Phe

SEQ ID NO:2

SEQUENCE LENGTH: 34

SEQUENCE TYPE: amino acid

TOPOLOGY: Linear

MOLECULE TYPE: Peptide

FEATURE:

LOCATION:1

OTHER INFORMATION:Xaa= Ser or Aib,

LOCATION:8

OTHER INFORMATION:Xaa= Met or naturally occurring hydrophobic amino acid

LOCATION:11

OTHER INFORMATION:Xaa= Leu, Ser, Lys or aromatic amino acid

LOCATION:12

OTHER INFORMATION:Xaa= Gly or D-amino acid

LOCATION:13

OTHER INFORMATION:Xaa= Lys or Leu

LOCATION:18

OTHER INFORMATION:Xaa= Met or naturally occurring hydrophobic amino acid

LOCATION:19

OTHER INFORMATION:Xaa= Glu or basic amino acid

LOCATION:21

OTHER INFORMATION:Xaa= Val or basic amino acid

LOCATION:23

OTHER INFORMATION:Xaa= Trp or 2-(1,3-ditionlan-2-yl) Trp

LOCATION:25

OTHER INFORMATION:Xaa= Arg or His

LOCATION:26

OTHER INFORMATION:Xaa= Lys or His

LOCATION:27

OTHER INFORMATION:Xaa= Lys , Gln or Leu

LOCATION:34

OTHER INFORMATION:Xaa= Phe or Phe-NH,

SEQUENCE DESCRIPTION:

Xaa Val Ser Glu Ile Gln Leu Xaa His Asn Xaa Xaa Xaa His Leu Asn
1 5 10 15

Ser Xaa Xaa Arg Xaa Glu Xaa Leu Xaa Xaa Xaa Leu Gln Asp Val
20 25 30

His Asn Xaa
34

Claims

1. A peptide or salts thereof represented by the formula

R_1-Val-Ser-Glu-Ile-Gln-Leu-R_2-His-Asn-R_3-R_4-R_5-
His-Leu-Asn-Ser-R_6-R_7-Arg-R_8-Glu-R_9-Leu-R_{10}-R_{11}-R_{12}-
Leu-Gln-Asp-Val-His-Asn-R_{13}

wherein R_1 represents Ser or Aib; R_2 represents Met or a naturally occurring hydrophobic amino acid; R_3 represents Leu, Ser, Lys or an aromatic amino acid; R_4 represents Gly or a D-α-amino acid; R_5 represents Lys or Leu; R_6 represents Met or a naturally occurring hydrophobic amino acid; R_7 represents Glu or a basic amino acid; R_8 represents Val or a basic amino acid; R_9 represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R_{10} represents Arg or His; R_{11} represents Lys or His; R_{12} represents Lys, Gln or Leu; and R_{13} represents Phe or Phe-NH_2; except that simultaneously R_1 consists of Ser, R_2 consists of Met, R_3 consists of Leu, R_4 consists of Gly, D-Ala or D-Pro, R_5 consists of Lys, R_6 consists of Met, R_7 consists of Glu, R_8 consists of Val, R_9 consists of Trp, R_{10} consists of Arg, R_{11} consists of Lys and R_{12} consists of Lys.

2. The peptide of claim 1 wherein a naturally occurring hydrophobic amino acid in R_2 and R_6 is Leu, Ile, Val, Phe or Trp.

3. The peptide of claim 1 wherein an aromatic amino acid in R_3 is Phe, β-naphthyl Ala, Trp or Tyr.

4. The peptide of claim 1 wherein a D-α-amino acid is D-Leu, D-Ile, D-Nle, D-Val, D-Ser, D-Ser(But), D-Abu, D-Thr, D-Nva, D-Met, β-naphthyl-D-Ala, D-Trp, D-Tyr, D-Lys, D-Lys(Fmoc), D-Phe or D-Asn.

5. The peptide of claim 1 wherein a D-α-amino acid is a neutral amino acid.

6. The peptide of claim 5 wherein neutral D-α-amino acid is D-Ser, D-Leu, D-naphthyl Ala, D-Trp, D-Asn or D-Tyr.

7. The peptide of claim 1 wherein basic amino acid in R_7 and R_8 is Arg, Lys, Asn or His.

8. The peptide of claim 1 wherein the peptide of the formula [1] simultaneously has 10 to 12 amino acids optionally selected from a group consisting of Ser for R_1, Met for R_2, Leu for R_3, Gly for R_4, Lys for R_5, Met for R_6, Glu for R_7, Val for R_8, Trp for R_9, Arg for R_{10}, Lys for R_{11}, Lys for R_{12} and Phe for R_{13}.

9. The peptide of claim 1 which is (Leu^{18})hPTH(1-34), (Aib^{1})hPTH(1-34), (Phe^{11})hPTH(1-34), (D-Trp^{12})-hPTH(1-34),(Leu^{8})hPTH(1-34)NH_2, (D-Tyr^{12})hPTH(1-34)NH_2, (D-Ser^{12})hPTH(1-34)NH_2, (D-Leu^{12})hPTH(1-34)NH_2, (3-(2-naphthyl-D-Ala^{12})hPTH(1-34)NH_2, (Ser^{11})hPTH(1-34)NH_2, (Phe^{11},Leu^{18})hPTH(1-34)NH_2, (Leu^{8},Phe^{11},Leu^{18})hPTH(1-34)NH_2, (Lys^{11})hPTH(1-34)NH_2, (Phe^{11})hPTH(1-34)NH_2, (Arg^{19,21})hPTH(1-34)-NH_2, (3-(2-naphthyl)-Ala^{11})hPTH(1-34)NH_2, (His^{26})hPTH(1-34)NH_2, (His^{25})hPTH(1-34), (Gln^{27})hPTH(1-34), (Arg^{19,21},2-(1,3-dithiolane-2-yl)Trp^{23})-hPTH(1-34)NH_2, (Leu^{27})hPTH(1-34) or (Lys^{11})hPTH(1-34).

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 477 885 A3**

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: **91116303.8**

(22) Date of filing: **25.09.91**

(51) Int. Cl.5: **C07K 13/00**, C07K 15/06, A61K 37/30

(30) Priority: **28.09.90 JP 257490/90**
06.09.91 JP 227232/91

(43) Date of publication of application:
01.04.92 Bulletin 92/14

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(88) Date of deferred publication of the search report:
27.10.93 Bulletin 93/43

(71) Applicant: **Takeda Chemical Industries, Ltd.**
1-1, Doshomachi 4-chome
Chuo-ku, OSAKA(JP)

(72) Inventor: **Nakagawa, Shizue**
23-709, 6 Nankhonaka 5-chome
Suminoe-ku, Osaka 559(JP)

Inventor: **Fukuda, Tsunehiko**
10-202, 9 Oharanonishisakaidani-cho
2-chome
Nishikyo-ku, Kyoto 610-11(JP)
Inventor: **Kawase, Masahiro**
28-108, Sasabe-daidononishi
Kawanishi, Hyogo 666(JP)
Inventor: **Yamazaki, Iwao**
9-21, Hibarigaokayamate 1-chome
Takarazuka, Hyogo 665(JP)

(74) Representative: **von Kreisler, Alek,**
Dipl.-Chem. et al
Patentanwälte
Von Kreisler-Selting-Werner,
Postfach 10 22 41,
Bahnhofsvorplatz 1
D-50462 Köln (DE)

(54) **Parathyroid hormone derivatives.**

(57) Disclosed are peptides and salts thereof represented by general formul

$$R_1\text{-Val-Ser-Glu-Ile-Gln-Leu-}R_2\text{-His-Asn-}R_3\text{-}R_4\text{-}R_5\text{-}$$

$$\text{His-Leu-Asn-Ser-}R_6\text{-}R_7\text{-Arg-}R_8\text{-Glu-}R_9\text{-Leu-}R_{10}\text{-}R_{11}\text{-}R_{12}\text{-}$$

$$\text{Leu-Gln-Asp-Val-His-Asn-}R_{13}$$

wherein R_1 represents Ser or Aib; R_2 represents Met or a naturally occurring hydrophobic amino acid; R_3 represents Leu, Ser, Lys or an aromatic amino acid; R_4 represents Gly or a D-α-amino acid; R_5 represents Lys or Leu; R_6 represents Met or a naturally occurring hydrophobic amino acid; R_7 represents Glu or a basic amino acid; R_8 represents Val or basic amino acid; R_9 represents Trp or 2-(1,3-dithiolane-2-yl)Trp; R_{10} represents Arg or His; R_{11} represents Lys or His; R_{12} represents Lys, Gln or Leu; and R_{13} represents Phe or Phe-NH_2; except that simultaneously R_1 consists of Ser, R_2 consists of Met, R_3 consists of Leu, R_4 consists of Gly, D-Ala or D-Pro, R_5 consists of Lys, R_6 consists of Met, R_7 consists of Glu, R_8 consists of Val, R_9 consists of Trp, R_{10} consists of Arg, R_{11} consists of Lys and R_{12} consists of Lys.

The parathyroid hormone (1-34) analogues are useful in hormone therapy.

EP 0 477 885 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number EP 91116303.8

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP - A - 0 341 963 (MERCK & CO. INC) * Claims 1-4 * --	1-9	C 07 K 13/00 C 07 K 15/06 A 61 K 37/30
A	EP - A - 0 293 159 (MERCK & CO. INC) * Claims 1-3 * --	1-9	
A	EP - A - 0 293 158 (MERCK & CO. INC) * Claims 1-4 * --	1-9	
A	DE - A - 3 428 942 (TOYO JOZO K.K.) * Claim 1 * ----	1-9	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 07 K 7/00
C 07 K 13/00
C 07 K 15/00
C 07 C 103/00
C 12 N 5/00
C 12 N 15/00
A 61 K 37/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	08-07-1993	SCHARF

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